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Dietary Supplementation with Olive Oil or Fish Oil and Vascular Effects of Concentrated Ambient Particulate Matter Exposure in Human Volunteers

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Short title: Olive oil and PM-induced endothelial dysfunction

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Abstract

Background: Exposure to ambient particulate matter (PM) induces endothelial dysfunction, a risk factor for cardiovascular disease. Olive oil (OO) and fish oil (FO) supplements have beneficial effects on endothelial function.

Objective: In this study we evaluated the potential efficacy of OO and FO in mitigating endothelial dysfunction and disruption of hemostasis caused by exposure to particulate matter (PM).

Methods and Results: Forty-two participants (58±1 year old) received either 3 gram/day of OO, FO, or no supplements (naïve) for 4 weeks prior to undergoing 2-hr exposures to filtered-air and concentrated ambient particulate matter (CAP) (mean 253±16 $\mu g/m^3$). Endothelial function was assessed by flow-mediated dilation of the brachial artery (FMD) pre-, immediately post- and 20 hours post-exposure. Levels of endothelin-1 and markers of fibrinolysis and inflammation were also measured. FMD was significantly lower after CAP exposure in the naïve (–19.4%; 95% CI: –36.4, –2.3 per 100 $\mu g/m^3$ CAP relative to baseline; p = 0.03) and FO groups (–13.7%; 95% CI: –24.5, –2.9; p = 0.01), but not in the OO group (–7.6%; 95% CI: –21.5, 6.3; p = 0.27). Tissue plasminogen activator levels were significant increased immediately after (11.6%; 95% CI: 0.8, 22.2; p = 0.04) and 20 hours after CAP exposure in the OO group. Endothelin-1 levels were significantly increased 20 hours after CAP exposure in the naïve group only (17.1%; 95% CI: 2.2, 32.0; p = 0.03).

Conclusions: Short-term exposure to CAP induced vascular endothelial dysfunction. OO supplementation attenuated CAP-induced reduction of FMD and changes in blood markers

associated with vasoconstriction and fibrinolysis, suggesting that OO supplementation may be an efficacious intervention to protect against vascular effects of exposure to PM.

Introduction

Epidemiological studies have demonstrated an association between exposure to ambient particulate matter (PM) at concentrations currently found in major metropolitan areas and a broad range of adverse cardiovascular outcomes (EPA 2009). A recent scientific statement from the American Heart Association concluded that short-term elevations in PM concentrations are capable of triggering acute coronary syndrome and stroke, worsening heart failure, and provoking arrhythmias among individuals with pre-existing heart disease (Brook et al. 2010).

Ambient PM is a complex and variable mixture of particles of varying size that are classified into coarse ($PM_{2.5-10}$; 2.5 μm < PM <10 μm in aerodynamic diameter), fine ($PM_{2.5;}$ PM < 2.5 μm) and ultrafine ($PM_{0.1}$; PM<0.1 μm) particles. In this study, research volunteers were exposed to $PM_{2.5}$ particles concentrated from the air in Chapel Hill, NC (CAP). Short-term controlled human exposure to $PM_{2.5}$ increases blood pressure (BP) and impairs endothelial function (Brook et al. 2009) in young healthy adults. Controlled exposure to diesel exhaust, a component of ambient PM, has been shown to affect fibrinolysis (Mills et al. 2005). A recent epidemiological study has associated long-term exposure to PM with decreased endothelial function (Krishnan et al. 2012). Moreover, $PM_{2.5}$ exposure in the Chapel Hill airshed was associated with immediate endothelial dysfunction in diabetic patients (Schneider et al. 2008). In addition, several controlled human exposure studies have suggested that air pollution exposure increases BP during (Brook et al. 2009; Urch et al. 2005) and immediately after PM exposure (Mills et al. 2005), and ambient $PM_{2.5}$ levels have been associated with higher BP in epidemiological studies (Auchincloss et al. 2008). However, the mechanisms of these vascular effects associated with exposure to ambient PM are still not well understood.

Endothelial dysfunction is a critical early event in the development of atherosclerosis (Ross 1993). Endothelial dysfunction is defined as an imbalance between vasodilating and vasoconstricting substances produced by and acting on the endothelium. Endothelial dysfunction results in elevated expression of chemokines, cytokines, and adhesion molecules that promote smooth muscle cell growth, platelet and leukocyte adhesion, thrombosis, and vascular inflammation. Endothelial dysfunction induced by air pollution exposure has been implicated as an important mechanism in the development of cardiovascular diseases (Brook et al. 2010; Krishnan et al. 2013). It is known that inflammation and oxidative stress mediate the adverse cardiovascular effects of air pollution exposure (Brook et al. 2010). Dietary supplements such as olive oil (OO) and fish oil (FO) have been shown to have anti-oxidant and anti-inflammatory effects (Moreno and Mitjavila 2003) that might offer protection against air pollution exposure. Consumption of OO, a principal component of the Mediterranean diet, has been shown to improve endothelial function and blood lipid profile (Zern and Fernandez 2005), decrease platelet aggregation (Delgado-Lista et al. 2011), and reduce vascular inflammation (Esposito et al. 2004). In particular, some components of OO, such as polyphenols (Moreno-Luna et al. 2012; Zern and Fernandez 2005) and oleic acid (Perona et al. 2006) have been shown to have beneficial effects on endothelial function. Therefore, OO supplementation is a potential candidate for use as a protective intervention against the adverse vascular effects of PM exposure. Dietary supplementation with FO has been shown to improve endothelial function in patients who smoke tobacco or who have elevated insulin, glucose or triglyceride levels (Duda et al. 2009a; Saravanan et al. 2010). However, the effects of OO and FO on vascular responses to air pollution exposure have not been examined specifically.

In the present study, we assessed the acute effects of controlled exposure to ambient air $PM_{2.5}$ on vascular endothelial function, blood pressure, and fibrinolysis and inflammation in healthy middle-aged human volunteers, and evaluated the efficacy of a 4-week regimen of dietary supplementation with OO or FO as a means to mitigate vascular effects induced by PM exposure.

Methods

Study participants

Forty-two participants ranging in age 50 to 72 years (mean 58±1 years) were enrolled in the study. None had a history of heart disease, uncontrolled hypertension, pulmonary disease, diabetes mellitus, hypercholesterolemia, or active allergy, and none had smoked for the past year. Participants were not taking n-3 or n-9 FA supplements, anti-inflammatory drugs (e.g. nonsteroidal anti-inflammatory drugs (NSAIDs)), or anti-oxidant supplements (e.g., beta-carotene, selenium, vitamin C, and vitamin E). All participants were instructed to avoid food sources of n-3 and n-9 FA for 6 weeks and refrain from using any NSAIDs for 2 weeks prior to each exposure. They were also asked to abstain from alcohol and caffeine and adhere to a low-fat diet for 24 hours before exposures. The Biomedical Institutional Review Board at the University of North Carolina-Chapel Hill and US Environmental Protection Agency approved the study protocol, recruitment materials, and consent forms. All study participants gave informed consent and received monetary compensation for their participation.

Study design

All exposures were conducted in the US Environmental Protection Agency Human Studies Facility on the medical campus of the University of North Carolina-Chapel Hill as described previously (Tong et al. 2012). At the end of 28 days of dietary supplementation, each participant was exposed first to filtered-air and then returned to HSF the next morning to undergo exposure to CAP on the next day. The participants were blinded to the exposure. The exposures were conducted at the same time of the day and same two days of the week. Participants were exposed for 2 hours through a face-mask in an exposure chamber in which temperature and humidity were controlled. They remained at rest in a seated position throughout the exposure.

The following procedures were performed on each participant beginning at approximately 8AM (two hours before chamber exposure to filtered-air): venous blood was collected; BP was recorded; and the diameter and flow-mediated dilation (FMD) of brachial artery was measured by ultrasound. The same procedures were repeated immediately following the 2-hr filtered-air exposure (post), and again the next morning at approximately 8 AM (follow-up). These latter measurements also served as the pre-exposure values for the CAP exposure. At approximately 10AM on the second day, participants were exposed to CAP for 2 hours. As with the filtered-air exposure, post and follow-up measurements were obtained immediately following CAP exposure and beginning at 8AM the next morning.

Dietary supplementation

As described previously (Tong et al. 2012), all participants were asked to restrict their intake of foods containing n-3 and n-9 FA for the 2 week period prior to and during the 4 weeks dietary

supplementation period. Thirteen participants received 3 gram/day (three 1 gram capsules daily) of OO, 16 participants received 3 gram/day (three 1 gram capsules daily) of marine derived n-3 FA (FO), and 13 participants were not supplemented (Naïve) for 28 days prior to the filtered-air exposure day. The participants were supplemented with OO or FO up to and including the days of exposure. FO and OO assignments were made using a randomized, double-blinded study design. Participants were also asked to keep 3-day food records during the 2nd and 4th weeks of the supplementation period to assess compliance with the dietary restrictions. Nutrition Data System for Research software (NDSR) (University of Minnesota) was used to analyze the food records and estimate intakes of nutrients that may confound n-3 FA. Each 1,000 mg OO capsule contained less than 1% of n-3 FA, with 73% of the FA content being oleic acid and 12% palmitic acid. Each 1,000 mg of FO capsule contained approximately 65% n-3 FA, consisting of 410 mg EPA and 274 mg of DHA. Pharmavite, LLC kindly provided the OO and FO supplements. All capsules used in this study were derived from single lots. The ratios of the major plasma fatty acids were measured at the end of the supplementation period to determine whether ratios were consistent with expectations for the FO and OO groups and the data has been reported previously (Tong et al. 2012).

Controlled exposure

CAP was generated as described previously (Tong et al. 2012) by drawing ambient air from above the roof of the Human Studies Facility (HSF) in Chapel Hill, NC and passing it through a two-stage aerosol concentrator capable of producing up to a 30-fold increase in particle number and mass. Air temperature and humidity were controlled inside the chamber. The concentration of particles delivered to the chamber varied with the level of naturally occurring ambient

particles in the Chapel Hill airshed at the time of the exposure. Particle mass and number concentrations at the chamber inlet were monitored continuously as described previously (Samet et al. 2009). A particle dilution system was used to limit the maximal particle concentration and prevent it from exceeding 600 $\mu g/m^3$ for > 6 min at any time during exposure. A face-mask was used to assure concordance between the PM concentration in the chamber and the concentration actually inhaled by the participant. Teflon filter samples were also obtained and analyzed for particle mass gravimetrically.

The 2-hr average fine/ultrafine CAP mass concentration in the chamber was 253±16 $\mu g/m^3$. The $PM_{2.5}$ concentration and particle number during the 2-hr CAP exposure and particle size is reported in Supplemental Material, Table S1 and Figure S1. It is estimated that 75% of particles were in the ultrafine range during exposure. The maximum 2-hr average inhalation exposure in this study is comparable to approximately 24 hr exposure to $PM_{2.5}$ at the current 24-hr National Ambient Air Quality Standard (NAAQS) (EPA 2012). No clinically significant events associated with CAP exposure were observed in any of the study participants.

Brachial artery ultrasound

As described previously (Guthikonda et al. 2007; Schneider et al. 2008), FMD was measured by brachial artery ultrasound in the University of North Carolina Hospitals Clinical Translational Research Center (CTRC) using a 12.5 MHz imaging probe interfaced with an ATL HDI 5000 ultrasound machine, or on-site at the U.S. Environmental Protection Agency HSF using a 15 MHz imaging probe interfaced with an Acuson Sequoia 512 ultrasound machine. The participants were transported from HSF to the UNC-CTRC by an E85-fueled vehicle. Baseline

images of the right brachial artery were captured at end-diastole. FMD was assessed after reactive hyperemia induced by inflating a pneumatic tourniquet applied distal to the antecubital fossa to a suprasystolic pressure for 5 min (Guthikonda et al. 2007). Hyperemic images were captured for 90 seconds following cuff deflation. Brachial arterial diameter was measured at baseline and at maximum dilation with customized software that utilizes edge-detection technology (Vascular Research Tools, Medical Imaging Application).

Blood pressure measurements

BP was measured using a validated ambulatory BP monitor (Oscar-2, SunTech) at 15-min intervals during 2 hours of exposure and at 30-min intervals before and after filtered air and CAP exposure. A single reading at each time point was acquired and used in the analysis.

Measurement of endothelin-1 and markers of fibrinolysis and inflammation

Venous blood was obtained 2 hours before, immediately after, and 20 hours after each exposure. Anti-coagulated plasma samples were stored at -80^{o}C until assayed in our laboratory using commercially available ELISA kits to quantify levels of fibrinolysis markers including tissue-type plasminogen activator (tPA) and plasminogen (Enzyme Research Laboratories), D-dimer and von Willebrand factor (vWF) (Diagnostisca Stago), plasminogen activator inhibitor-1 (PAI-1) (DakoCytomation), inflammatory markers including fibrinogen (Diasorin), IL-6, IL-8, TNFα, C-reactive protein (CRP) (Meso Scale Discovery), vascular inflammatory markers including vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Meso Scale Discovery), and vasoconstrictor endothelin-1 (Bachem Group).

Statistical analysis

To evaluate changes between CAP and filtered-air exposures within participants, as well as differences between the OO, FO, and naïve groups, we used a two-factor (supplement and CAP concentration) mixed effects model with a participant-specific random intercept. Changes within individuals were evaluated at two time points separately: immediately and approximately 20 hours after exposure to CAP and filtered-air, denoted "Post" and "Follow-up" respectively. Prior to the analysis, we normalized all "Post" and "Follow-up" outcomes to their pre-exposure baseline (Post/Pre, Follow-up/Pre) to control for day-to-day variability. Exposure variable PM was treated as continuous and changes between CAP and filtered-air exposures were expressed as percent point differences per 100 $\mu g/m^3$ increase in CAP concentration relative to baseline, with the associated 95% confidence interval. To test differences in baselines between three groups and between exposures we used a two factor mixed effects model (exposure= (pre-filtered-air, pre-caps), group = (OO, FO, naïve). There were more females than males in each dietary supplement group in this study and the ratio of females to males varied (9:4, 12:4, and 11:2 in OO, FO, and naïve groups, respectively). However, the great majority of female participants (29 of 32) were post-menopausal, and only 4 were on hormone replacement therapy (Table 1). To examine the influence of gender on responses to CAP and the outcomes we conducted sensitivity analyses for selected endpoints by adjusting for gender as a confounder, and by restricting analyses to women only. We did not have sufficient power to evaluate effect modification of associations with CAP, or of differences according to supplement group, by gender. We also examined the sensitivity of results with respect to medication usage by excluding 5 participants taking statins or ACE inhibitors. R statistical software (version 2.15.0; R

Development Core Team) was used for analysis and a *p* value of less than 0.05 was considered significant.

Results

Table 1 presents characteristics of the research participants. Age, BMI, smoking history, medication usage, BP, serum glucose and lipids did not differ statistically among the groups. Before starting supplementation with OO or FO, or no supplements (naïve), all participants reported low dietary intakes of foods rich in n-3 and n-9 FA (Tong et al. 2012). Participants showed good compliance with their adherence to the dietary restriction and OO or FO supplementation schedule (Tong et al. 2012).

Endothelial function

The average baseline diameter and FMD of brachial artery measured before, immediately after, and 20 hours after CAP exposure are presented in Table S2 (see Supplemental Material). Compared with the naïve group, the mean value of FMD measured prior to the filtered-air exposure was higher in the FO group (7.19±0.88 vs. 6.57± 0.65; p = 0.08) when analyzed using a two-factor mixed effects linear model for changes in baselines between exposures (filtered-air, CAP) and supplemental groups (OO, FO, naïve). There were no significant differences among the pre-filtered-air exposure baseline diameter mean values in the OO, FO, and naïve groups.

The mean percent difference from filtered-air control in FMD and baseline brachial artery diameter (BAD) per 100 $\mu g/m^3$ increase in CAP concentration immediately after and 20 hours after CAP exposure is shown in Table 2. FMD was significantly lower immediately after CAP exposure in the naïve group (-19.4% average decrease relative to baseline per 100 $\mu g/m^3$ increase

in CAP concentration; CI: -36.4%, -2.3%; p=0.03) and also in the FO group (-13.7%; 95% CI: -24.5%, -2.9%; p=0.01) (Table 2 and Figure 1). In the OO group, however, exposure to CAP resulted in a smaller non-significant decrease in FMD (-7.6%; 95% CI: -21.5%, 6.3%; p=0.27). In the FO group, FMD 20 hours after CAP exposure was still significantly lower than baseline (-21.5%; 95% CI: -37.3%, -5.6%; p=0.01). Differences between the groups were not significant. Reduction in FMD was persistent after adjusting for confounding by gender as well as in the 'female-only' analysis (see Supplemental Material, Table S3). There was also no significant difference in baseline diameter of brachial artery either immediately or 20 hours after CAP exposure in any of the groups (Figure 1 and Table 2).

Blood pressure

There was no significant difference in BP before filtered-air exposure between the OO, FO, and naïve groups (Table 1). There were statistically non-significant increases in systolic BP 30 min after CAP exposure in the naïve group (+2.5 mmHg relative to filtered-air exposure/100 $\mu g/m^3$ CAP concentration; p=0.09) and 60 min after exposure in the FO (+2.7 mmHg; p=0.06) and OO (+2.1 mmHg; p=0.22) groups. Diastolic BP was significantly increased 30 min after exposure in the naïve group (+2.1 mmHg relative to filtered-air exposure/100 $\mu g/m^3$ CAP concentration; p=0.04) and 60 min after exposure in the FO (+2.1 mmHg; p=0.008) and OO (+2.1 mmHg relative to filtered-air exposure/100 $\mu g/m^3$ CAP concentration; p= 0.03) groups.

Blood markers of fibrinolysis

The average concentrations of blood markers of fibrinolysis at baseline, immediately post-, and 20 hours post exposure is presented in Table S4 (see Supplemental Material). In all three

supplement groups we observed higher levels of plasma PAI-1 in the morning (8-9am) and lower levels in the afternoon (1-2pm). The observed variation is consistent with previous reports (Paschos and FitzGerald 2010; Rudnicka et al. 2007) and may indicate diurnal variation in levels of plasma PAI-1, however this study was not designed to further examine this hypothesis. Baseline tPA concentration (ie., before filtered air exposure) was significantly lower in the OO group compared with the naïve group, and baseline PAI-1 and plasminogen concentrations were significantly lower in the OO and FO groups compared with the naïve group (Supplemental Material, Table S4), possibly due to effects of OO and FO supplementation (Mehta et al. 1988; Perez-Jimenez et al. 2006).

In the OO group, the average plasma concentration of tPA increased immediately after CAP exposure in the OO group (11.6%; 95% CI: 0.8%, 22.2%; p=0.04), and this increase persisted for 20 hours, at which time there was a 10.9% increase (95% CI: 0.1%, 21.8%; p=0.05) for this group (Table 2 and Figure 2). In addition, plasma D-dimer levels were 11.6% lower (95% CI: -22.6%, -0.5%; p=0.04) 20 hours after CAP exposure in the OO group.

The association between CAP exposure and tPA in the OO group was robust to adjustment for confounding by gender, but changed from positive to slightly negative when the four men in the OO group were excluded from the analysis, suggesting substantial differences in association between CAP and tPA among males and females in the OO group (Supplemental Material, Table S3). The association between CAP and D-dimer levels in the OO group was similar after adjusting for gender and after excluding men from the analysis (Supplemental Material, Table S3). When 5 participants taking either statins or ACE inhibitors were excluded from the OO

group, plasma D-dimer levels were no longer significantly decreased 20 hours after CAP exposure (-0.7% per 100 ug/m^3 increase in CAP; 95% CI:-10.4%, 9%; p=0.88). This suggests that these medications may have influenced effects of CAP on plasma D-dimer levels, though differences due to chance cannot be ruled out because of small sample size and potential for selection bias. Excluding participants using these medications did not have obvious impact on other associations in the OO group, nor did it appear to influence associations in FO or naïve groups.

Endothelin-1 level

Blood concentrations of endothelin-1, a potent vasoconstrictor that could mediate the vascular effects of CAP exposure. There was an increase in plasma endothelin-1 levels in the naïve group (17.1%; 95% CI: 2.2%, 32.0%; p=0.02) 20 hours after CAP exposure, but not in the OO group (Figure 3 and Table 2).

Markers of inflammation

Pre-filtered-air exposure levels of IL-8, ICAM-1, and VCAM-1 were significantly lower in the OO and FO groups relative to the naïve group, while TNFα levels were higher in the OO and FO groups relative to the naïve group (Table S4, see Supplemental Material). There were no significant changes in concentrations of plasma markers of inflammation after CAP exposure in the OO, FO, and naïve groups (Table 2).

Discussion

In the present study, acute exposure to CAP was associated with decreased endothelial function, as measured by FMD, in middle-aged volunteers that did not receive a dietary supplement. We

further show that dietary supplementation with OO, but not FO, appeared to blunt the endothelial dysfunction induced by exposure to CAP. We also observed that the concentrations of blood fibrinolysis marker tPA increased immediately after CAP exposure in participants supplemented with OO, an effect that persisted for 20 hours after exposure. In addition, plasma endothelin-1 levels were increased 20 hours after CAP exposure in the un-supplemented group but not in the OO group. Taken together, these findings suggest that supplementation with OO provides potentially beneficial vascular effects by conferring protection against CAP-induced endothelial dysfunction in middle-aged adults.

Endothelial dysfunction is an important factor in the development of hypertension and atherosclerosis that is associated with an increased risk of coronary heart disease. Specifically, endothelial dysfunction induced by air pollution exposure has been implicated as an important mechanism in the development of cardiovascular diseases (Brook et al. 2010; Krishnan et al. 2013). Mechanistically, oxidative stress and inflammation are two mechanisms that have been associated with the adverse health effects of air pollution exposure. It has been suggested that anti-oxidant components in certain food groups can limit oxidative damage and thereby protect endothelial function (Moreno and Mitjavila 2003). The results presented in this study suggest that dietary supplementation with OO may prevent deleterious effects of CAP exposure on vascular function and might, therefore, represent a practical approach to reduce the mortality and morbidity of cardiovascular diseases associated with PM exposure.

There was a smaller CAP-induced reduction in FMD in participants supplemented with OO compared to FO supplemented and un-supplemented participants in this study, supporting a role

for OO in protecting vascular function from PM-induced health effects. The oleic acid in OO has antioxidant and anti-inflammatory properties (Perona et al. 2006), and OO supplementation has been shown to improve endothelial function and lipid profile (Zern and Fernandez 2005) and lower the risk of coronary heart disease events (Estruch et al. 2013; Hertog et al. 1995). Two months of dietary supplementation with OO was associated previously with improved endothelial function and a decrease in BP in a study of young women with mild hypertension (Moreno-Luna et al. 2012). Furthermore, in a clinical trial in healthy young adults, 16 weeks of supplementation with 4 gram/day of OO led to an increase in FMD (Singhal et al. 2013). Our finding suggests that 4 weeks of dietary OO supplementation attenuate PM-induced reductions in FMD, possibly due to effect of OO protect against the adverse effects of PM exposure on endothelial function.

We also observed in this study that on average participants in the OO supplementation group had significant changes in the levels of tPA to CAP exposure while those in FA and naïve groups did not. There was a significant increase in plasma levels of tPA immediately after the CAP exposure and this effect persisted 20 hours post exposure in the OO group. tPA is a serine protease that catalyzes the conversion of plasminogen to plasmin, the major enzyme responsible for the breakdown of thrombi. On the other hand, D-dimer was significantly reduced 20 hours after CAP exposure in the OO group, though the effect was no longer present after 8 participants using statins and ACE inhibitor medications were excluded from the OO group, which suggests that the association may have been influenced by medication use in these participants. D-dimer is a fibrin degradation product present in the blood after a thrombus is degraded by fibrinolysis. We have previously reported that the levels of D-dimer were increased following controlled exposure

of young healthy volunteers to ultrafine CAP (Samet et al. 2009). The increase in tPA level suggests that the supplementation with OO may activate the fibrinolysis system that protects against thrombosis induced by CAP exposure. However, it is difficult to reconcile this mechanism with the simultaneous decrease in D-dimer level observed in these participants. One possibility is that OO could be decreasing prothrombotic factors that were not measured in this study.

In addition, we observed that plasma levels of endothelin-1 were increased following CAP exposure in the un-supplemented group but not in participants supplemented with OO or FO. Several studies have shown increased plasma endothelin-1 levels after controlled exposure of human volunteers to diesel exhaust (Peretz et al. 2008), and following exposure to urban ambient levels of air pollution in adults (Liu et al. 2009) and children (Calderon-Garciduenas et al. 2007), suggesting that increased concentrations of this potent vasoconstrictor is a consequence of endothelial damage resulting from air pollution exposure. The attenuation of CAP-induced endothelin-1 levels in the OO group shown in this study suggests beneficial effects of OO supplementation on vascular tone and endothelial function. However, increases in BP following CAP exposure were comparable among the OO, FO and un-supplemented groups. It has been suggested that the CAP-induced BP elevation may be caused by autonomic imbalance(Brook et al. 2009). We have previously demonstrated that CAP exposure alters the sympatho-vagal balance (Samet et al. 2009; Tong et al. 2012) by increasing the sympathetic input to the cardiovascular system which could result in increased BP.

It has been hypothesized that FO-mediated enhancement of endothelial function is a potential strategy by which the population can be protected from the adverse effects of PM. Previous studies have demonstrated that FO (EPA and DHA) improve systemic arterial compliance in individuals with dyslipidemia (Nestel et al. 2002), and improve endothelial function in individuals with dyslipidemia, heart failure, and diabetes (Duda et al. 2009b; Saravanan et al. 2010). However, trials of FO supplementation in healthy volunteers have shown inconsistent results (Khan et al. 2003; Singhal et al. 2013). One study found that 8 months of FO supplements improved endothelial function in middle-aged men and women (Khan et al. 2003). By contrast, 16 weeks of DHA supplementation had no effect on indices of endothelial function in a study of healthy young volunteers (Singhal et al. 2013). In general, studies have shown that EPA and DHA levels in blood are not linked to FMD in normal healthy adults (Egert and Stehle 2011; Leeson et al. 2002; Sanders et al. 2011; Saravanan et al. 2010; Singhal et al. 2013). Consistent with these reports, in our present study we did not observe a significant benefit of 4 weeks of FO supplementation on CAP-induced reduction in FMD.

The mechanism by which OO supplementation attenuates CAP-induced vascular effects was not investigated in this study. It is possible that OO supplementation increases the bioavailability of plasma nitrites/nitrates, which in turn improves endothelial function (Moreno-Luna et al. 2012). In the present study, OO supplementation appeared to blunt the CAP-induced reduction in FMD, consistent with an effect of OO on the bioavailability of NO. Another function of OO is to promote anti-inflammatory effects in the vasculature. It has been shown that oleic acid, a principal bioactive component of OO, inhibits endothelial activation by suppressing inflammatory responses in vitro (Carluccio et al. 1999). It is also possible that OO

supplementation increases resistance of HDL and LDL to oxidation (Moreno et al. 2003; Sola et al. 1997) induced by CAP exposure.

In order to avoid potential variations in fatty acid equilibrium times and particle washout periods, this study did not use a standard crossover design involving a randomized exposure to air and CAP. In addition, conclusions derived from the small number of participants included in the study may not be applicable to the population as a whole. Furthermore, the modest sample size and the number of secondary endpoints measured which could inflate the significance of the findings are additional statistical limitation. In spite of these limitations, as the first controlled exposure study of this type, the present study reports statistically significant differences between the response to CAPs and filtered-air under each of the three dietary regimens for several important outcomes. These findings provide the basis for a future cohort study focusing on between-group comparisons in order to confirm and further evaluate OO supplementation as a protective intervention against the adverse health effects of PM inhalation.

Conclusion

In this study we observed that a 2-hr exposure to CAP impaired vascular endothelial function for up to 20 hours, and that dietary supplementation with OO, but not FO, attenuated the FMD reduction induced by CAP exposure. Further, supplementation with OO also seemed to alter blood markers associated with fibrinolysis and vasoconstriction in PM-exposed human volunteers. These data, therefore, suggest that OO supplementation should be further examined as a possible intervention to protect against the adverse vascular effects of air pollution exposure.

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Table 1. Characteristics of the participants before dietary restriction and supplementation.

Characteristics	Olive oil (n=13)	Fish oil (n=16)	naïve (n=13)
Sex (male/female)	4/9	4/12	2/11
Age (years)	59.3 ± 1.1	57.4 ± 1.4	57.8 ± 1.3
Post-Menopausal	8	11	10
Race (White/Black)	11/2	10/6	10/3
Body Mass Index (m^2/kg)	26.3 ± 1.3	27.6 ± 1.1	24.9 ± 1.2
Systolic blood pressure (mmHg)	123 ± 3	122 ± 3	121 ± 2
Diastolic blood pressure (mmHg)	77 ± 2	77 ± 2	76 ± 3
Heart rate (bpm)	71 ± 3	74 ± 2	66 ± 3
Cholesterol			
Total (mg/dl)	214 ± 7	201±12	210 ± 10
LDL (mg/dl)	125 ± 9	117 ± 10	120 ± 9
VLDL (mg/dl)	19 ± 3	19 ± 2	18 ± 2
HDL (mg/dl)	70 ± 6	64 ± 4	71 ± 6
Triglyceride (mg/dl)	94 ± 14	97 ± 10	93 ± 8
Glucose (mg/dl)	93 ± 2	88 ± 3	92 ± 2
WBC ($x10^3$/µl)	5.46 ± 0.39	5.53 ± 0.25	5.21 ± 0.36
RBC ($x10^6$/µl)	4.76 ± 0.17	4.45 ± 0.12	4.54 ± 0.07
Platelets ($x10^3$/µl)	223.3 ± 12.2	245.9 ± 13.6	250.4 ± 13.6
Neutrophils (%)	55.3 ± 2.7	55.9 ± 2.9	50.5 ± 4.7
Lymphocytes (%)	34.6 ± 2.5	34.9 ± 2.9	29.8 ± 3.2
Smoking history (n)			
Non-smokers	11	11	13
Ex-smokers	2	5	0
Current smokers	0	0	0
Medications (n)			
Statin	2	1	0
β-adrenergic receptor blockers	0	0	0
ACE inhibitors	3	2	0
Anti-depressant	1	2	0
NSAIDs	0	0	0
HRT	1	2	1

Data are means ± SEM. LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; WBC, white blood cells; RBC, red blood cells; ACE inhibitors, angiotensin converting enzyme inhibitors; NSAIDs, non-steroid anti-inflammatory drugs; HRT, hormone replacement therapy. Age, BMI, medication usage, blood pressure, blood cell counts, serum glucose and lipids were not significantly different among the groups (ANOVA, $p>0.05$).

Table 2. Mean percent point difference per 100 μg/m^3 increase in CAP concentration relative to baseline (pre-filtered-air exposure) measured immediately after (post) and 20 hours after (FU) CAP exposure in each group.

Endpoints	OO (n=13)		FO (n=16)		naïve (n=13)	
	Post-CAP	FU	Post-CAP	FU	Post-CAP	FU
BAU						
FMD	-7.6(-21.5, 6.3)	-5.4(-25.8, 15.1)	-13.7(-24.5, -2.9)*	-21.5(-37.3, -5.6)*	-19.4(-36.4, -2.3)*	-17.9(-43.0, 7.2)
BAD	0.2(-0.5, 0.9)	-0.3(-1.4, 0.8)	0.2(-0.3, 0.8)	-0.2(-1.0, 0.7)	-0.4(-1.3, 0.4)	0.8(-0.5, 2.2)
Blood Markers						
ET-1	-4.7(-14.8, 5.4)	-10.0(-22.1, 2.1)	-0.4(-7.8, 7.0)	1.4(-7.9, 10.8)	8.3(-3.7, 20.2)	17.1(2.2, 32.0)*
tPA	11.6(0.8, 22.2)*	10.9(0.1, 21.8)*	2.2(-6.4, 10.9)	1.9(-7.2, 11.1)	-0.50(-12.8, 11.7)	1.2(-11.7, 14.0)
PAI-1	0.1(-6.6, 6.8)	-4.4(-15.5, 6.6)	-0.6(-5.6, 4.4)	-2.3(-10.9, 6.3)	6.3(-1.7, 14.4)	6.2(-7.4, 19.7)
D-dimer	-4.1(-24.0, 15.8)	-11.6(-22.6, -0.5)*	-1.9(-16.5, 12.6)	1.4(-7.2, 10.0)	-16.9(-40.4, 6.7)	-2.9(-16.5, 10.6)
Plasminogen	-6.7(-16.3, 2.9)	-5.3(-14.3, 3.6)	-3.1(-10.4, 4.2)	1.2(-5.7, 8.2)	-2.0(-13.6, 9.6)	-3.7(-14.6, 7.3)
vWF	4.5(-2.5, 11.7)	0.4(-20.7, 21.5)	0.3(-4.9, 5.5)	7.0(-9.4, 23.4)	-0.1(-8.5, 8.3)	-0.6(-26.5, 25.2)
Fibrinogen	1.0(-3.8, 5.8)	0.4(-4.0, 4.8)	0.2(-3.4, 3.8)	2.7(-0.7, 6.2)	5.0(-0.8, 10.8)	2.3(-3.1, 7.8)
CRP	0.7(-24.4, 25.9)	3.7(-14.4, 21.8)	-1.7(-20.7, 17.2)	-5.0(-19.1, 9.1)	-2.5(-32.8, 27.7)	9.5(-12.7, 31.7)
ICAM-1	0.8(-29.1, 30.7)	-0.5(-17.4, 16.3)	-1.8(-24.3, 20.6)	-1.7(-14.8, 11.3)	0.3(-35.6, 36.3)	7.3(-13.3, 27.9)
VCAM-1	0.1(-28.4, 28.7)	-0.7(-20.0, 18.6)	-2.4(-23.9, 19.0)	-3.2(-18.2, 11.8)	2.1(-32.1, 36.5)	5.5(-18.1, 29.2)
IL-6	-1.4(-12.3, 9.3)	-1.0(-12.3, 10.2)	-3.9(-12.2, 4.3)	-1.7(-10.5, 7.0)	-4.7(-17.7, 8.4)	-9.2(-23.0, 4.6)
IL-8	-1.1(-5.7, 3.4)	1.2(-4.0, 6.5)	-0.1(-3.6, 3.4)	0.3(-3.8, 4.4)	3.7(-1.8, 9.2)	-3.3(-9.8, 3.1)
TNFα	1.8(-1.5, 5.2)	0.2(-3.5, 4.0)	-0.04(-2.6, 2.5)	0.6(-2.3, 3.5)	-0.3(-4.3, 3.7)	-3.9(-8.6, 0.6)

Endpoints are summarized as mean and 95% confidence intervals. OO, olive oil group; FO, fish oil group; CAP, concentrated ambient air pollution particles; FU, follow-up; BAU, brachial artery ultrasound; FMD, flow-mediated dilatation; BAD, baseline diameter of brachial artery; ET-1, endothelin-1; tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibator-1; plasm, plasminogen; vWF, von Willebrand factor; CRP, c-reactive protein; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion protein 1; Il-6, interleukin 6; Il-8, interleukin 8; TNFα, tumor necrosis factor α.*$p<0.05$, compared to pre-CAP.

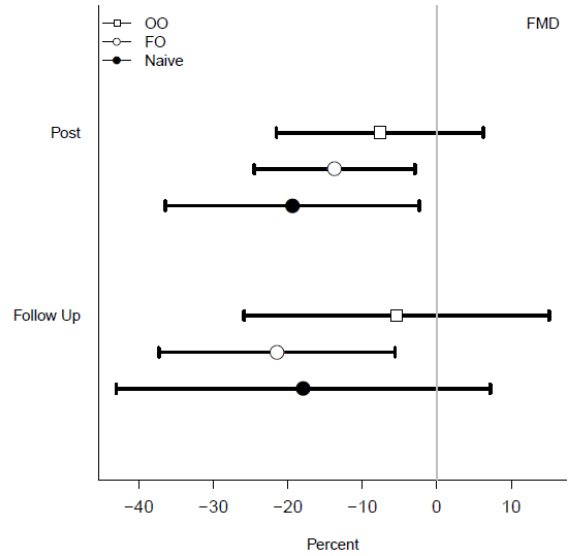
Figure Legends

Figure 1. Effect of concentrated ambient particle (CAP) exposure on parameters of brachial artery ultrasound. Flow-mediated dilation (FMD) (**A**) and baseline diameter (**B**) of the brachial artery were measured by ultrasound before, immediately following exposure to filtered-air and CAP (post), and again the next morning (follow-up) as described in METHODS. Squares represent average percent point differences between CAP and filtered-air exposure per 100 $\mu g/m^3$ increase in CAP relative to the baseline and bars indicate 95% confidence intervals in the OO group; open circles represent endpoints measured in the FO group; solid circles represent endpoints measured in the naïve group.

Figure 2. Effect of concentrated ambient particle (CAP) exposure on plasma markers of fibrinolysis. Blood was collected before, immediately following exposure (post) to filtered air and CAP, and again the next morning (follow-up) and markers of fibrinolysis, tPA (**A**) and D-dimer (**B**) were assayed by ELISA kits described in METHODS. Squares represent average percent point differences between CAP and filtered-air exposure per 100 $\mu g/m^3$ increase in CAP relative to the baseline and bars indicate 95% confidence intervals in the OO group; open circles represent endpoints measured in the FO group; solid circles represent endpoints measured in the naïve group.

Figure 3. Effect of concentrated ambient particle (CAP) exposure on plasma endothelin-1 levels. Blood was collected before, immediately following exposure (post) to filtered air and CAP, and again the next morning (follow-up) and assayed for endothelin-1 using an ELISA kit as described in METHODS. Squares represent average percent point differences between CAP and filtered-air exposure per 100 $\mu g/m^3$ increase in CAP relative to the baseline and bars indicate 95% confidence intervals in the OO group; open circles represent endpoints measured in the FO group; solid circles represent endpoints measured in the naïve group.

A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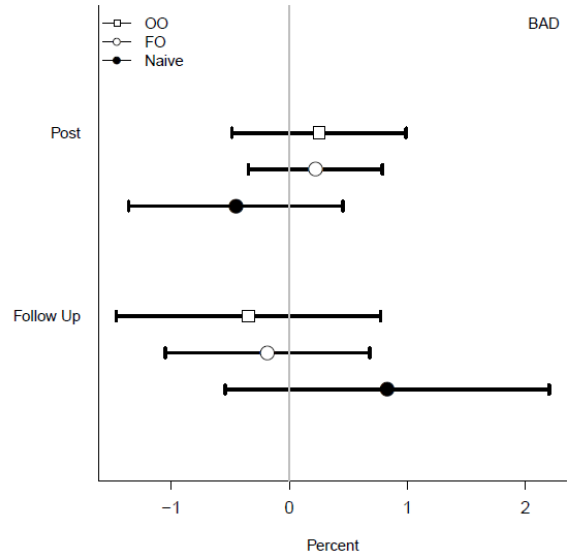
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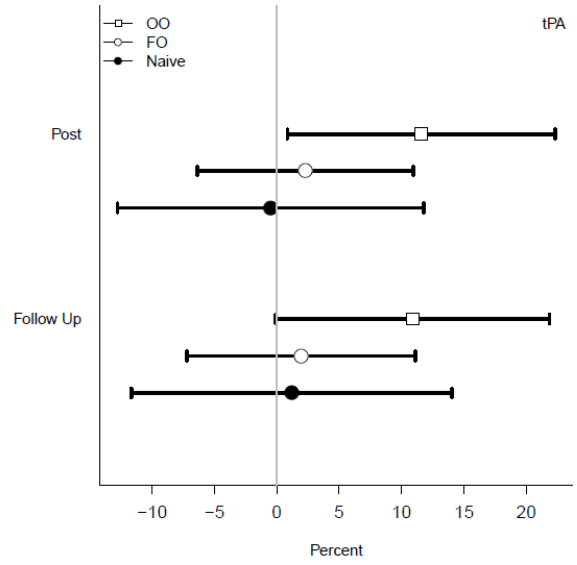
Figure 1

A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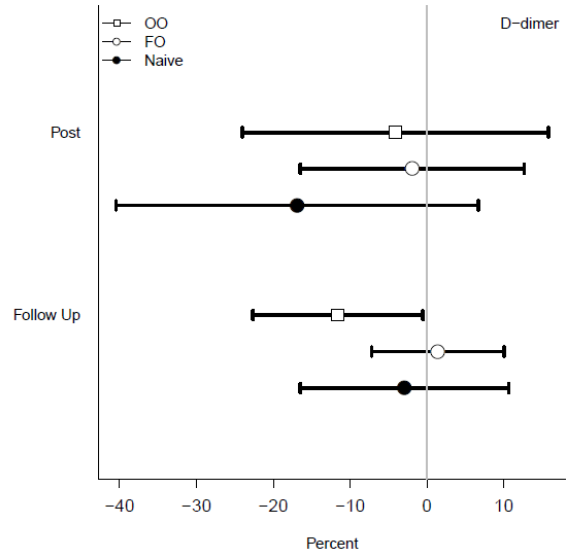
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Figure 2

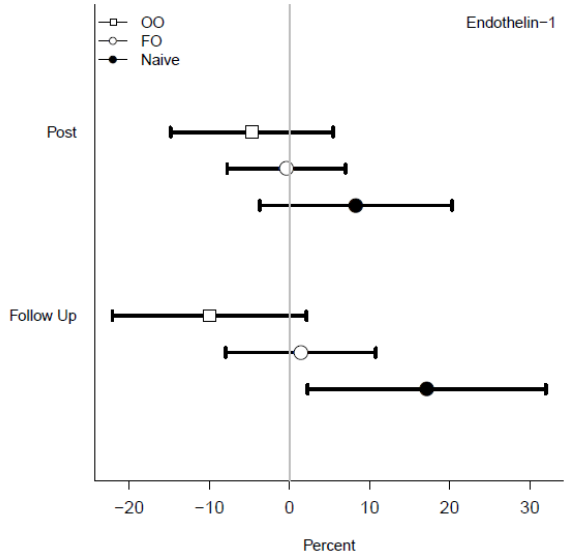
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Figure 3